

Research Note

Effects of Suspension in Emulsified Wiener or Incubation in Wiener Packages on the Virulence of *Listeria monocytogenes* Scott A in Intragastrically Inoculated A/J Mice[†]

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ABSTRACT

Several outbreaks of listeriosis have been associated with contamination of wieners and other ready-to-eat meat products. In this study, we addressed the question of whether emulsification in, or growth on, wieners triggers a response in the listerial cells that makes them more virulent or protects them against the harsh environment of the gastrointestinal tract in mice. Our results indicate that *Listeria monocytogenes* Scott A grows poorly, if at all, in one brand of commercially prepared wieners inoculated with 5×10^3 to 5×10^6 CFU per package and incubated at 15°C. Neither *L. monocytogenes* Scott A emulsified in a slurry of homogenized wieners nor recovered from wiener package fluid after a 7-day incubation at 15°C were more virulent when inoculated into the stomachs of A/J mice than *L. monocytogenes* Scott A grown in brain heart infusion broth. These findings suggest that the ability of *L. monocytogenes* Scott A to cause systemic infection following introduction into the gastrointestinal tract was not improved by incubation with wieners or suspension in a meat matrix.

Listeria monocytogenes is a ubiquitous gram-positive bacterium that is frequently present on food of plant and animal origin (12, 16). Furthermore, it can establish itself within certain niches in the environment of a food processing plant, despite appropriate sanitation efforts (26). Although the number of cases of listeriosis are much lower than those of other foodborne bacterial pathogens, such as *Campylobacter* or *Salmonella*, it ranks higher in mortality, causing up to an estimated 500 deaths in the United States each year (3). The threat posed by *L. monocytogenes* is amplified by its ability to grow over a broad temperature range, including the ability to multiply at refrigeration temperatures (12). As a result, contamination of a food product with even small numbers of *L. monocytogenes* conceivably can result in a significant inoculum if the food is properly stored at refrigeration temperatures for extended periods of time. It is the combination of its propensity to cause severe infections and its ability to multiply at refrigeration temperatures that makes *L. monocytogenes* a food safety threat, particularly in ready-to-eat (RTE) foods (12, 22, 25). As a result, the United States has very stringent regulations regarding the presence of detectable *L. monocytogenes* in

RTE food products. This “zero tolerance” policy for *L. monocytogenes* in RTE foods and attendant sampling regimens for the bacterium in RTE foods has led to numerous and costly product recalls (3–6, 10, 25).

Several large outbreaks of listeriosis have been associated with serotype 4b strains of *L. monocytogenes* (12). Several of these outbreaks were associated with contamination of a RTE meat product. In 1998, an outbreak involving wieners from a processing plant in Michigan caused 101 cases of listeriosis and 20 deaths (4). Two independent outbreaks associated with sliced turkey meat occurred in 2002 (1, 5, 6). In the first, 16 of 44 attendees at a catered party developed acute febrile gastroenteritis following ingestion of contaminated deli meat (10). In the second outbreak, there were 46 culture-confirmed cases, 7 deaths, and 3 stillbirths or abortions (1). One of the unanswered questions related to these outbreaks and others is whether the environmental signals encountered by *L. monocytogenes* while growing on a food affect its expression of virulence determinants (5, 6, 13, 17, 20, 24). If this occurs, it might allow *L. monocytogenes* to survive better within the harsh environment of the gastrointestinal tract or adhere to and translocate across the intestinal epithelium, from which it can disseminate to internal organs in which it multiplies and causes systemic disease. An alternative question is whether being enmeshed in the matrix of a food product protects the listeriae from the harsh acidic conditions they encounter as they pass through the gastrointes-

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[†] Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

tinal tract (21), thus increasing the probability that listeriae can translocate across the intestinal epithelium.

To address these questions, we used a model of gastrointestinal listeriosis in genetically susceptible A/J mice (9). In this study we sought to determine whether growth of *L. monocytogenes* on a RTE meat product (i.e., wieners) or suspension in emulsified wiener would increase the severity of infection in this mouse model of gastrointestinal listeriosis.

MATERIALS AND METHODS

Preparation of *L. monocytogenes*. An aliquot of log phase cells of *L. monocytogenes* Scott A (serotype 4b; originally obtained from the culture collection of the Food Research Institute, University of Wisconsin–Madison) that was stored at -70°C was thawed, inoculated into brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, Md.), and incubated overnight with shaking at 37°C as described previously (9). Following this, the bacteria were harvested by centrifugation ($1,000 \times g$ for 20 min), resuspended in the same volume of BHI containing 20% glycerol, and stored at -70°C as 1-ml aliquots. Before each experiment, an aliquot was thawed, inoculated into 50 ml of BHI broth, and incubated overnight at 37°C with shaking at 150 rpm. The optical density of the bacterial suspension was read with a spectrophotometer (Smart Spec 3000, Bio-Rad, Hercules, Calif.), and the number of *L. monocytogenes* (CFU) was extrapolated from a standard growth curve. To prepare the inoculum for mice, appropriate dilutions were made in sterile phosphate-buffered saline (PBS) to achieve the desired bacterial concentration. The number (CFU) in the inoculum was verified by plating on blood agar (Trypticase soy agar with 5% bovine blood; BBL, Becton Dickinson, Franklin Lakes, N.J.) and counting colonies after 24 h of incubation at 37°C .

Inoculation of *L. monocytogenes* into wiener packages. Commercial packages of wieners were purchased and kept at 4°C until used in an experiment and were not held beyond the use-by date. The ingredients for the mixed-meat product were listed as “mechanically separated chicken, meat ingredients (pork, beef) water, corn syrup, salt. Contains 2% or less of flavorings, dextrose, sugar, potassium lactate, paprika, sodium phosphates, sodium erythrobate, sodium nitrite; may contain sodium ascorbate.”

For each experiment, two wieners were aseptically removed from the original packaging and placed in a sterile bag of the same characteristics. Four milliliters of the desired numbers of stationary-phase *L. monocytogenes* Scott A (suspended in PBS) was added to the bags, which were then vacuum sealed and incubated at 15°C for up to 7 days. To estimate the number of viable *L. monocytogenes*, samples (0.5 ml) of fluid from each of three bags were aseptically removed at daily intervals, diluted in saline, and plated on blood agar. The plates were incubated at 37°C , the colonies counted, and the numbers (CFU per milliliter) estimated for each package.

Inoculation of mice. Female inbred A/J mice were obtained (Harlan Sprague-Dawley, Indianapolis, Ind.) at 5 to 6 weeks of age and housed under microisolator caps at the University of Wisconsin School of Veterinary Medicine animal care facility. Mice were acclimated for 1 to 2 weeks in this facility before being used in an experiment. Mice received food and water ad libitum until 5 h before an intragastric (i.g.) inoculation experiment, at which time food was removed from the cage. This was done to prevent mechanical blockage of the delivery of the listerial inoculum into the stomachs of mice that were engorged with mouse chow, pre-

venting aspiration of the inoculum into the lungs. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (0.75 to 1 mg/25 g mouse). Once mild sedation occurred, the listerial inoculum was introduced (in a total volume of 0.2 ml) via a 1.5-in., 24-gauge stainless steel feeding needle attached to a 1-ml syringe. Six mice per experimental group were used for each experiment.

For experiments in which mice were inoculated with *L. monocytogenes* Scott A incubated in wiener packages, the inoculated packages were prepared as described previously. On the day before the scheduled inoculation of mice, fluid was aseptically removed (0.5 ml) from each of two bags, diluted in PBS, and plated separately onto blood agar to determine viable *L. monocytogenes* (CFU). The next day, the package fluid was appropriately diluted in PBS to obtain the desired number of *L. monocytogenes* cells for inoculation of mice. Control mice received the same number of *L. monocytogenes* Scott A cells grown in 4 ml of BHI broth, incubated for approximately 4 days at 15°C . Numbers of viable listerial cells in the inocula were confirmed by plating on blood agar.

For experiments in which mice were inoculated with *L. monocytogenes* Scott A suspended in a slurry of homogenized wiener, Scott A was grown overnight in BHI broth, and the numbers of viable cells were estimated by optical density. The stationary phase listerial cells were washed and resuspended in PBS to the desired concentration. A portion of a wiener was homogenized in a blender with PBS at a ratio of approximately one part wiener to four parts PBS. The meat solids were allowed to settle to the bottom of the tube (e.g., “slurry”); the upper fluid phase of the wiener homogenate was designated as the supernatant. The supernatant was removed and placed in a separate sterile tube. The desired concentration of *L. monocytogenes* were then added to the slurry or supernatant and used to inoculate mice i.g. as described before. Control mice received the same number of *L. monocytogenes* Scott A cells from the same BHI broth cultures that were washed and diluted in PBS. The number of *L. monocytogenes* (CFU) in the various inocula was confirmed by plating on blood agar.

Recovery of *L. monocytogenes* from the tissues of infected mice. At the desired time points postinoculation, mice were humanely euthanized by asphyxiation with CO_2 , followed by exsanguination according to an animal use protocol approved by the University of Wisconsin School of Veterinary Medicine Animal Care and Use Committee. In some experiments, blood was collected into a syringe containing sodium citrate as anticoagulant. The blood was then serially diluted in sterile saline and plated in duplicate (0.1 ml) on blood agar, and the plates were incubated at 37°C to detect bacteremia with *L. monocytogenes*. Next, the abdominal cavity was aseptically opened, and portions of the spleen and liver were removed. These tissues were weighed in sterile weigh boats and placed in sterile glass tissue grinders (Wheaton Science Products, Millville, N.J.) that contained 1 ml of cold sterile saline. The tissues were then manually homogenized to a liquid state, diluted in sterile saline, and plated in duplicate on blood agar. The cecum and its contents were also removed, homogenized in saline as indicated above, and plated onto modified Oxoid agar (Alpha Biosciences, Baltimore, Md.), which would allow for ready recognition of the listeria colonies. The plates were allowed to dry at room temperature and then were incubated at 37°C for 24 h. The colonies were then counted (mean \pm standard error of the mean [SEM], log CFU of *L. monocytogenes* per wet weight gram of tissue). Representative colonies were confirmed as *L. monocytogenes* by beta-hemolysis on Trypticase soy agar with 5%

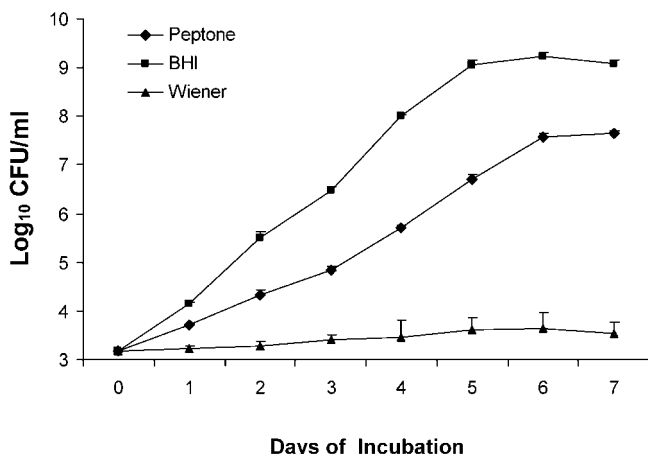


FIGURE 1. Growth curves of *L. monocytogenes* Scott A inoculated (10^3 CFU) into packages of wieners, BHI broth, or proteose peptone and incubated at 15°C . Samples were aseptically removed at daily intervals and plated on modified Oxoid agar. Results are the mean \pm SEM from one representative experiment (three replicates for each growth condition).

bovine blood after 48 h of incubation at 37°C , and tumbling motility was confirmed when grown overnight at room temperature on blood agar, resuspended in isotonic saline, and viewed microscopically in a hanging drop at $\times 400$ magnification.

Statistical analysis. Data were analyzed with an analysis of variance with GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, Calif.). If a significant *F*-value was obtained ($P < 0.05$), then the Tukey-Kramer test was performed to determine whether the means of treatment groups differed from controls. Statistical significance for all comparisons was set at $P < 0.05$.

RESULTS

We initially performed growth curve experiments in which packages of Brand A wieners were experimentally inoculated with relatively low numbers (10^3 CFU/ml) of *L. monocytogenes* Scott A. The inoculated packages were then incubated at a temperature that would represent temperature abuse (15°C). We observed little or no increase in the numbers of viable *L. monocytogenes* during a 7-day incubation period, compared with a greater than 4-log multiplication of *L. monocytogenes* Scott A BHI broth or peptone broth incubated at the same temperature (Fig. 1). However, it should be noted that only one brand of wieners was used in this study, and their formulation could differ from that of other brands, including wieners associated with earlier listeriosis outbreaks. Thus, we cannot make inferences regarding the growth of *L. monocytogenes* in wiener packages beyond the strain of *L. monocytogenes* and brand of wieners used in this study.

Because *L. monocytogenes* did not multiply in the wiener packages, we could not easily obtain sufficient numbers of listeriae to inoculate mice with a challenge dose (i.e., 10^6 CFU per mouse) that would result in systemic infection. We attempted to circumvent this problem in several ways. The first was to grow *L. monocytogenes* in BHI broth and then suspend a portion of the culture ($\sim 10^7$ CFU) in a slurry of homogenized wiener or in the supernatant resulting from centrifugation to remove the meat solids (see

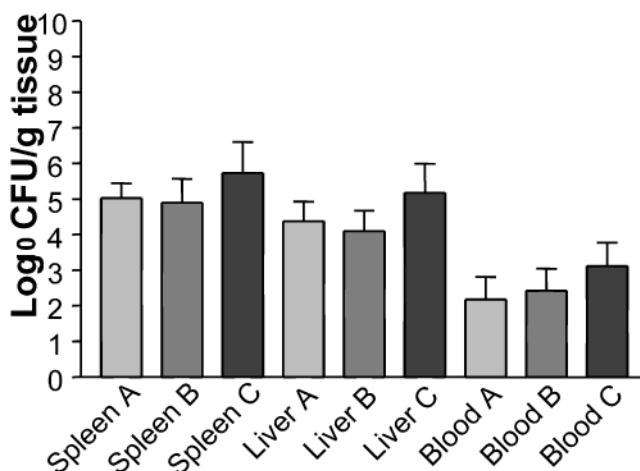


FIGURE 2. *L. monocytogenes* Scott A was suspended in BHI (A), a slurry of emulsified wiener (B), or the supernatant of the latter (C) following centrifugation to remove meat solids. The resulting listerial suspensions were then inoculated i.g. into anesthetized mice, as described in "Materials and Methods" (10^6 CFU in 0.2 ml per mouse). The mice were euthanized 3 days later, blood was collected, and portions of the spleen and liver were removed. The tissues were homogenized in PBS, and dilutions of the tissue homogenates and blood were plated onto modified Oxoid agar. The plates were incubated at 37°C , and the numbers (CFU) were determined. The results are the mean \pm SEM of a single experiment (five mice per group). For each of the organs sampled, none of the treatment groups differed statistically from one another ($P > 0.05$).

"Materials and Methods"). In these experiments, we found no evidence that immersion of *L. monocytogenes* in either the homogenized wiener or the supernatant of the homogenate significantly increased the severity of infection compared with *L. monocytogenes* Scott A suspended in PBS (Fig. 2).

Finally, we addressed this question by intentionally inoculating wiener packages with larger numbers of *L. monocytogenes* (10^7 CFU/ml) and incubating for 5 days at 15°C . Although the listeriae still failed to multiply in the packages (data not shown), we were able to recover enough organisms from the package fluid to inoculate mice via the intragastric route (10^6 listeriae per mouse). In this experiment, we also found no evidence that exposure of *L. monocytogenes* to the environmental conditions found within a package of wieners had any effect on the resulting severity of infection in our mouse model, compared with mice inoculated with *L. monocytogenes* grown in BHI at 15°C (Fig. 3).

DISCUSSION

Several large outbreaks of listeriosis have been associated with ingestion of RTE meat products that were contaminated with *L. monocytogenes* (1, 4–6, 10). The environment contained within a package of wieners exposes *L. monocytogenes* to potential stressors such as pH, salt concentration, sodium lactate, sodium diacetate, phenols, and lactic acid bacteria that might initiate a stress response via its environmental sensing systems (2, 7, 11–13, 17, 23). As examples, exposure to glucose or acidic conditions can alter

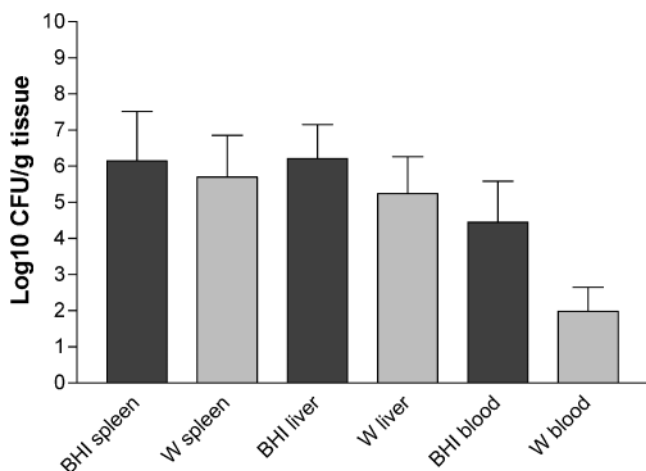


FIGURE 3. *L. monocytogenes* Scott A was inoculated (10^7 CFU) into commercial packages of wieners and incubated for 5 days at 15°C . The package fluid was recovered and used to inoculate anesthetized A/J mice i.g., as described in "Materials and Methods". Control mice received *L. monocytogenes* grown in BHI broth at 15°C for 4 days. The mice were euthanized 3 days later, blood was collected, and portions of the spleen and liver were removed. The tissues were homogenized in PBS, and dilutions of the tissue homogenates and blood were plated on modified Oxoid agar. The plates were incubated at 37°C , and the numbers (CFU) were determined. The results are the mean \pm SEM from a single experiment (five mice per group). For each of the organs sampled, the two treatment groups were not statistically different from one another ($P > 0.05$).

the subsequent heat resistance of *L. monocytogenes* cells, and incubation of *L. monocytogenes* cells in frankfurter exudate increases their resistance to simulated gastric fluid (2). Our hypothesis at the initiation of this study was that exposure to the environment present in a package of RTE meat (i.e., wieners) would trigger a stress response that would activate or potentiate the expression of regulatory molecules that control the expression of important virulence determinants of *L. monocytogenes* (7, 11, 17, 20). Alternatively, we also considered the possibility that the complex matrix provided by emulsified wiener might protect *L. monocytogenes* during its journey through the harsh environment of the gastrointestinal tract and, by so doing, increase the number of organisms that survived and were capable of attaching to and translocating across the intestinal epithelium (15). The results of this study do not support these hypotheses. We obtained no evidence that growth or suspension of *L. monocytogenes* in wieners alters the virulence of the organism or protects it to such an extent that it is better able to cause systemic infection via the gastrointestinal tract of mice, compared with *L. monocytogenes* grown in BHI. However, it should be noted that we used a single commercial source of wieners, for which the formulation might not reflect that of other brands of wieners or of wieners implicated in earlier outbreaks.

This study used a mouse infection model that we have described previously (9). A strength of this model is that we observe systemic infection and death following i.g. inoculation of numbers of *L. monocytogenes* (10^6 CFU) that

might be found in a contaminated food product. The elegant study of Lecuit et al. (14) provided evidence for the relationship between *L. monocytogenes* expression of the surface protein internalin and E-cadherin on the basolateral surface of intestinal epithelial cells. Because mouse E-cadherin does not bind internalin, it was suggested that the mouse was not a desirable model for gastrointestinal listeriosis. However, it should be noted that even in an optimal internalin–E-cadherin system (i.e., human E-cadherin transgenic mice or guinea pigs), these authors needed to inoculate 10^{10} *L. monocytogenes* to cause lethal infection. Because *L. monocytogenes* contains many other known virulence factors and a wealth of surface proteins that might influence attachment and invasion, we believe it is premature to exclude the mouse as a model for investigating the pathogenesis of gastrointestinal listeriosis.

However, several limitations to our experimental design should be noted. First, we have examined only one strain of *L. monocytogenes* (Scott A) in this study. Although strain Scott A was associated with a foodborne disease outbreak (12), its properties might not be representative of other foodborne or clinical disease isolates of *L. monocytogenes*. It has been reported that some strains of *L. monocytogenes* can survive better in experimentally inoculated wiener packages than Scott A (19). Whether other strains might differ in their virulence following experimental inoculation into wiener packages, slurry, or supernatant was not examined in this study. Second, the organisms were grown at 15°C , which is higher than one would find in a typical case of temperature abuse. However, in previous studies, we reported that the temperature at which *L. monocytogenes* is grown in BHI broth has no significant effect on its virulence in the gastrointestinal tract (8, 9). Also, we have not seen an appreciable difference in recovery of *L. monocytogenes* from wiener packages maintained at 4°C versus 10°C (27). Nor does the inclusion of lactate in the product formulation substantially affect heat resistance of *L. monocytogenes* inoculated into wiener packages (18). However, we cannot exclude the possibility that the mouse inoculation results obtained following incubation of *L. monocytogenes* in wiener packages might be affected by incubation temperature. Nonetheless, the results of this study suggest that factors other than simply being suspended in the complex matrix of a RTE meat product is required to alter the resistance of mice to gastrointestinal listeriosis.

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